LA-UR-85-3568

CONF-8510225--1

Los Alamos National Laboratory is operated by the University of California for the United States Department of Energy under contract W-7405-ENG-36.

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DE86 002403

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SUBMITTED TO: Proceedings of the Twenty-Fourth Hanford Life Sciences Symposium
Richland, Washington; October 21-24, 1985

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DNA ANALYSIS OF EPITHELIAL CELL SUSPENSIONS

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ABSTRACT

The skin and lung are two of the major interfaces between man and his environment. he epithelial cells in these tissues are targets for chemically-induced tumorigenesis. A ommon step during in vivo development of skin and lung tumors is basal cell migration and yperplasia followed by differentiation; this reaction is also an important defense echanism. Cell proliferation can be monitored by flow cytometric (FCM) analysis which lso allows separation of the various cell populations. Of particular interest were the hort-term effects of shale oils on skin, in view of previously documented long-term ffects, including production of skin tumors. Cell suspensions of skin were obtained by nzymatic and mechanical disruption of intact tissues from control animals and animals xposed by topical application (skin painting) of several crude oils. DNA analysis of hese cell suspensions labeled with mithramycin provide determination of percentages of ells in the G_1, S and G_2M phases of the cell cycle. Data acquired showed differences rom control animals occurring as early as 7 days after treatment and persisting through l days afterwards. There was histological evidence of erythema and hyperplasia in shale il-exposed skins. FCM analysis of DNA content in shale-oil-exposed skin cells showed an ncreased percentage of cycling cells (28-31%, compared to 16% for controls), plus vidence of aneuploidy. Similar data from a positive control group (abraded skin) showed ncreased percentages of cycling cells, but no aneuploidy. Additionally, the hale-oil-exposed group, when compared to a standard petroleum-exposed group, had

significantly increased percentages of cycling cells. This early indication of differing response to different complex mixtures was also seen in long-term skin exposures to these compounds. As a supplement to the study of DNA analysis of skin cell suspensions, we developed a technique to disperse the epithelial lining of the trachea. Similar analytical techniques were applied to tracheal cell suspensions from ozone-exposed rats. Using DNA content measurements of mithramycin-stained fixed cells, we failed to demonstrate induction of cell proliferation. However, suspensions of Hoescht-stained viable cells showed increased percentages of cycling cells, a finding which was supported by histological examination of sections of ozone-exposed tracheas. In this manner, it is possible to study early cellular responses in cell populations of two major organ systems following acute, in vivo exposures to a variety of compounds.

INTRODUCTION

Tumors of the skin and lung are recognized as possible adverse health effects ollowing exposure to the fluid or gaseous phases of a wide range of complex organic aterials. The increased explcitation of commercial and novel organic compounds requires onitoring for potential carcinogenic activity. Current more stringent regulations overning most products of the energy and chemical industries have forced assessment of raditional testing methods.The latent period between initial exposure and the appearance f cancerous lesions is often long in humans; thus, it would be inappropriate to wait for vidence of human disease. Organic compounds can be tested in a variety of *in vivo* and *in itro* assay systems. However, animal experiments are lengthy, costly, and impractical for esting all compounds, while *in vitro* systems lack the cellular and tissue interactions ften important in the induction of neoplastic lesions.

A practical approach to this problem is to identify a short-term, *in vivo* effect ıich may reflect the long-term induction of either skin or lung tumor. This screening ›uld be followed by further animal studies concentrating on the suspect materials. To ıis end, we are employing FCM methods to identify abnormal changes in the DNA and ·ll-cycle characteristics of skin cells, initially, and, as an additional model, tracheal ›ithelial cells. Previous studies in our laboratory (Holland and Wilson, 1980) have ıown that several shale oils induce neoplastic changes following chronic skin exposure ıile either of two standard petroleums elicit markedly diminished tumorigenic responses. ›llowing extensive dose-response studies to define long-term toxicity and tumorigenicity, · have now concentrated our attention on short-term (21 days) effects following acute ·in exposures. The present study assesses evidence of increased percentages of cycling ·lls and the presence of aneuploid DNA following exposure to compounds that have been ıown to have high biological activity.

MATERIALS AND METHODS

Skin Cell Suspensions

For this study we used 10-week-old female C3H mice (Jackson Labs, Bar Harbour, Maine) housed five per cage in polyethylene, filter-top cages, bedded with aspen chips, and fed rodent chow (TEKLAD , Winfield, Iowa) and chlorinated water ad libitum. One week prior to treatment, all experimental animals, except controls, had the hair on their backs clipped using small animal clippers (Oster, Milwaukee, Wisconsin) equipped with a #40 blade. On the day of treatment, the animals were clipped again and 100 microliters of each test oil was applied by micropipette. A positive control group (abraded) was included to assess natural repair. Table 1 shows the protocol for the study. Animals were sacrificed by cervical dislocation at 1,4,7,10,14 or 21 days post exposure. The treated area was clipped to remove regrown hair, gently washed with wet gauze. and a dipilatory (Nair®, New York City, New York) was applied. After eight minutes, the dipilatory was washed off using gauze sponges and warm water. This area was then cut away from the back musculature and placed epidermis down and the dermis/connective tissue side up on a dissecting board. Using a #11 scalpal, the excess fat and connective tissue were scraped off leaving only the epidermis. Figure 1 is a photomicrograph of a section of control skin showing the morphology of the tissue prior to suspension. The tissue was weighed then finely minced with iris scissors. Collagenase (Worthington, Freehold, NJ), dissolved in phosphate buffered saline (PBS) with calcium and magnesium to a concentration of 0.8%, was added to the tissue; this suspension was then incubated at 37° C for 30 minutes in a shaking, dry incubator. After 30 minutes, the suspension was removed from the incubator and aspirated in and out of a 15 gauge needle connected to a 10-ml syringe (10X). The suspension was returned to the incubator for an additional 30-min enzyme digestion. At the end of the incubation periods, the suspension was again mechanically dispersed by aspiration through a 15-gauge needle and 10-mL syringe, followed by aspirations (5X) through 300 micron and 70 micron stainless steel microsieves (Millapore, Bedford, Massachusetts). The suspension was centrifuged (200 X g/10 min); the supernatent was then carefully removed by aspiration. The pellet was resuspended in 2 mL alpha-MOPS, a complete medium

(Gibco, Grand Island, NY), with 10% fetal calf serum (Rehatuan, Armour Pharmaceutical, Kankakee, Illinois). The cells were counted on a hemacytometer and viability was determined by Trypan Blue dye exclusion. After counting and viability assay, the cells were removed from the medium by centrifugation (200 X g/10 min) and washed (2 X) in saline. Final suspension was in 2 mL of normal (0.9%) saline to which 4 mL of ethyl alcohol was added to fix the cells for flow analysis of DNA content.

Tracheal Cell Suspensions

Male Fischer-344 rats (200-250 gm each) were randomly assigned to either the control or experimental groups. Control animals were maintained in the colony in polyethylene, filter-top cages (two animals per cage) and fed pelletized rodent feed (TEKLAD , Windfield, Iowa) and chlorinated water ad libitum. Experimental animals were housed the same as controls, but were exposed acutely, by nose-only inhalation, (Smith et al., 1981) to 15 ppm ozone. Following exposure for 30 minutes the rats were returned to the colony. At 2,3,4,7,8 and 10 days after exposure, randomly-selected animals were sacrificed by intraperitoneal injections of Nembutal (sodium pentobarbitol, 25 mg). The larynx, trachea and extrapulmonary bronchi were removed from the lungs. Tracheas were cannulated with a short, blunt 15-gauge stainless-steel needle secured by ligature at the larynx. The lumen of the tracheas were flushed with 5 mL alpha-MOPS (Gibco, Grand Island, NY). Hyaluronidase (Sigma, St. Louis, Missouri) was dissolved in alpha-MOPS at a concentration of 0.2% prior to instillation into the lumen which was sealed at the distal end. The inflated tracheas were placed in petri dishes containing 2 mL alpha-MOPS and placed in an incubator (no CO_2, 37° C) for 15 min. After the incubation period, the tracheas were flushed with 5 ml alpha-MOPS. During the enzyme incubation periods, tracheal flushes were maintained on ice. The lumens were refilled with cytochalasin B (Sigma, St. Louis, Missouri) also dissolved in alpha-MOPS to a concentration of 0.2%. Cytochalasin B is not readily soluable in aqeous vehicles, so the stock was dissolved in DMSO and dilutions into medium were made from this. The cytochalasin B-inflated tracheas were returned to the incubator (no CO_2, 37° C) for 1 hr. At the end of the incubation, the tracheas were flushed with another 5 mL alpha-MOPS. Finally, the tracheal lumens were filled with

pronase (Sigma, St. Louis, Missouri) which had been dissolved in alpha-MOPS (concentration = 0.2%) and similarly incubated for 30 min. As before, the lumen was flushed with 5 mL alpha-MOPS at the end of the incubation period. The pooled samples of all incubations and flushes from individual animals were centrifuged (200 X g/10 min.), washed two times in normal saline, then resuspended in normal saline (2 mL). Each sample was assayed by hemacytometer innumeration for number of cells harvested along with percent viable cells using Trypan Blue dye exclusion. Like the skin samples, these were fixed by the addition of 4 mL of ethyl alcohol, then stored in the refrigerator awaiting flow analysis.

DNA Analysis

Fixed cell samples, either skin or trachea, were centrifuged to remove the alcohol fix. Samples were spun (200 X g/10 min), the supernate decanted, and the cell pellet resuspended in magnesium buffered saline containing mithramycin (Pfizer, Groton, CT) at a concentration of 0.5%. The analysis of fluorescence intensity associated with of mithramycin-stained DNA (Crissman and Tobey, 1974) was done on the National Flow Resource B-D FACS instrument (funded by NIH and DOE). Following analysis, the data were reanalyzed by computer to determine the number of events measured in the G_1, S, and G_2M peak regions associated with resting and cycling cells. In addition to this method, we analysed viable tracheal cells using Hoescht 33342 (Calbiochem, La Jolla, CA) staining of DNA coupled with multiparameter analysis of several light scatter characteristics (in press).

Data Analysis

Cell yields were tabulated for all samples of skin and trachea. In each case, there were five animals/sacrifice date. The means and standard deviations of these values were calculated and their statistical significance of difference determined by Student-t test for p values < 0.05. Percent cycling cells was determined by gated analysis of the DNA content distributions based on the total number of events occurring in the resting and cycling populations divided into the number of events occurring in only the S, G_2M region. Additional analysis of two-dimensional plots of fluorsence intensity versus light scatter parameters provided percentages of cycling cells in Hoescht (HO) stained preparations.

RESULTS

Skin Cells

Cell Numbers. There was no consistent pattern in the number of cells recovered per milligram of tissue, although the numbers obtained from the abraded skins were generally lower than those of the cage controls (Table 2).

Viability: There was no consistent pattern in the percentages of viable cells in the skin cell suspensions from exposed and control groups. However, the final values obtained on day 21 for the API- and PHRR-treated animals were significantly lower than the abraded skins and the cage controls (Table 2).

Cell Cycle Analysis. The number of cycling cells in the abraded skins were significantly elevated throughout the post-treatment analysis period. The shale oil-treated skins produced significantly increased percentages of cycling cells starting 7 days after treatment that remained elevated throughout the recovery period. The API reference petroleum produced an increased percentage of cycling cells starting 7 days after treatment but subsiding by 21 days post-exposure. Comparison of the shale oil and the reference petroleum showed a significant increase in the percentage of cycling cells present in the shale oil-exposed group over the reference petroleum-exposed animals. Additionally, some of the distributions of DNA-content from shale oil-exposed animals showed a shoulder on the DNA peak associated with the resting cell population (Fig. 1). This excess peak was not present in control, abraded, or API#1-exposed animals.

Histology. At t_1, all the skins appeared similar with the exception of the PHRR treated which showed a marked erythema (Fig.1). At t_2 the cage controls and API #1-treated skins were similar in appearance with no gross abnormalities while the PHRR-treated skins showed a pronounced hyperplasia. The abraded skins possessed an intermediate histological pattern. The erythema in the PHRR-treated animals persisted up to t_7 after which it was no longer apparent. The hyperplasic response remained in the PHRR and abraded skins for the duration of the 21-day test period.

Tracheal Cells

Cell Number. No significant differences were apparent between any of the exposed groups or the control animals (Table 3).

Cell Viability. The viability of the cells recovered starting at two days after exposure showed significant reductions compared with the control animals (Table 3).

DNA-Cell Cycle Analysis. DNA-content analysis using mithramycin staining of fixed tracheal cells failed to show any significant increases or decreases from controls in the percentages of cycling cells present (Table 3). However, HO staining of viable cells produced evidence of significantly increased percentages of cycling cells as early as two days after exposure and markedly increased by t_{10} (Table 4).

Histology. The tracheas from early post-exposure periods (t_1, t_2) showed a generalized loss of ciliated cells (Fig. 2). Tracheas from t_4 and t_6 were not distinguishable from control tissues. This is in contrast to the tracheas from t_8 and t_{10} which showed marked hypertrophy of the epithelial cells and secretory cell hyperplasia.

DISCUSSION and CONCLUSIONS

The epithelial cells of the skin and lung are targets for environmental and/or industrial carcinogens. By producing single-cell suspensions of these two tissues, we have initiated studies of the capacity of various compounds to induce cell proliferation. The techniques reported here for harvesting cell suspensions produce good cell yields with high viability. This is particularly evident with the tracheal cell preparation which produces yields several times greater than any currently reported in the literature (Terzaghi, 1980; Wu, 1982). The suspension of cells from these two tissues in either saline or complete medium allows analysis of DNA content as well as determination of cell surface and density characteristics by flow cytometry. Increased cell turnover in these tissues has been associated with both normal injury repair and tumor production (Krueger and Shelby, 1981; Furstenberger et al., 1980; Kennedy, 1978). Bauer et al. (1980) demonstrated flow cytometric analysis of cell kinetics using biopsy specimens of human

epidermis and described methods for quantitation of DNA content and calculations of percentages of cells in S and G_2M. Nettesheim et al. (1982) used DNA-content measurements of cultured tracheal cells, grown from isolated tracheal rings and exposed in-vitro to chemical carcinogens, to demonstrate a correlation between the induction of aneuploidy and tumorigenesis. They showed that induction of cell proliferation was not sufficient to predict tumorigenesis, unless it was coupled with the appearance of aneuploid DNA.

Using extensions of these observations, our studies concentrated on cellular effects initiated in vivo following an acute exposure to two toxic agents known to produce epithelial cell death followed by repopulation from basal cell types. Many previous studies involving testing for the ability of materials to induce cell proliferation in the epidermis or trachea revolved around the use of long-term, in vivo tumorigenesis assays or isolated tissue fragments maintained in culture. For the skin studies reported here, mice were painted with two types of crude oils, one a natural crude petroleum crude and the other a crude shale oil. In previous studies employing long-term exposure to multiple dose levels, these two complex hydrocarbon mixtures had been shown to be tumorigenic but with considerable difference in potency (Fig.3). The shale oil produced 100% skin tumors in less than 500 days while the natural petroleum produced only 5% tumors in the same time span. In the present study, using an acute exposure to the oils and a survey period of 21 days, we demonstrated induced hyperplasia, increased percentages of cycling cells and evidence of aneuploidy (Fig. 4). In the positive control group (abraded skin), fewer cells were harvested but the percentages of cycling cells were elevated and there was no evidence of aneuploid DNA.

Previous studies of high and low ozone exposure had shown ozone to be cytotoxic to ciliated epithelium, an effect which was reversed over a period of days by basal cell proliferation and differentiation resulting in repopulation of the tracheal lining Menzel, 1984). This model was, therefore, different from the skin model as there is no reported correlation between ozone inhalation and tumor production. However, the model has value for expressing DNA-content changes reflecting cell proliferation following acute

inhalation of a known toxicant. While the DNA-content analysis of fixed, mithramycin-stained cells failed to show significant differences from control values, analysis of HO-stained viable cells clearly showed an increased percentage of cycling cells. There was no evidence of aneuploid DNA in tracheal cell suspensions stained with either mithramycin or HO. Histological changes following ozone exposure included an initial loss of ciliated cells followed by hypertrohic and hyperplasic responses.

It was our objective with these studies to begin developing model systems to study cellular response following acute, in vivo exposures to simple compounds and complex mixtures. These are short-term assays that can demonstrate initial mechanisms of cell injury resulting in proliferation of cells types which can be quantitatively measured. We have produced cell suspensions amenable to these DNA-content measurements that could also be used for in vitro studies of functional impairment or could be used as transplant tissue to study tumorigenesis. It was of additional interest that these two organ systems could be concurrent target tissues for many organic materials.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the expertise of Mark Wilder and Robb Habbersett for flow cytometric analysis of the samples, Ellie Margiotta for cell suspension preparation, and Nathan Belcher of Pfizer Chemicals for the donation of the fluorochrome Mithramycin through the National Flow Cytometry Resource. This work was partially funded by NIH grant P41-RR0135-02 and performed under the auspices of the U.S. Department of Energy Contract W-7405-ENG-36.

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TABLE 1

DISCRIPTION OF MATERIALS AND TREATMENT PROTOCOLS FOR SKIN- AND TRACHEA-EXPOSED ANIMALS

Group		Dose	Material
Skin			
	Control	---	Maintained in colony
	Abraded	---	Shaving 1 wk prior to t_o, then again on t_o plus brushing shaved area
	API #1	100 µL	American Petroleum Institute Reference Oil #1
	PHRR	100 µL	Shale Oil from Pilot Project at Anvil Points, Co.
Trachea			
	Ozone	15 ppm	30 min, nose-only inhalation

TABLE 2

COMPARISONS OF CELL YIELDS AND PERCENTAGES OF CYCLING CELLS FOR SKIN CELL SUSPENSIONS OF CONTROL AND TREATED GROUPS

Group	Elapsed Time (Days)	Number of Cells per mg Tissue ($\times 10^3$)	Percent Cycling Cells	Percent Viability
Control		7.3 ± 0.9	16.3 ± 3.1	75.8 ± 6
Abraded	1	3.1 ± 0.6[a]	23.2 ± 10.9	88.8 ± 1.6[a]
	4	4.7 ± 1.7[a]	21.6 ± 4.6[a]	89.6 ± 4.2[a]
	7	4.9 ± 2.1a	25.4 ± 7.6[a]	79.6 ± 2.1
	10	2.7 ± 1.3[a]	23.0 ± 3.1[a]	84.8 ± 7.8[a]
	14	6.5 ± 2.0	35.5 ± 8.5[a]	80.6 ± 4.5
	21	4.5 ± 1.7[a]	23.6 ± 4.8[a]	80.6 ± 3.4
API#1	1	8.8 ± 1.3[a]	18.6 ± 0.9	88.0 ± 2.6[a]
	4	7.2 ± 2.8	17.0 ± 2.2	89.6 ± 4.1[a]
	7	9.2 ± 0.6[a]	20.4 ± 1.5[a]	81.4 ± 5.0
	10	2.8 ± 1.4[a]	24.0 ± 4.2[a]	80.8 ± 1.9
	14	4.7 ± 1.9[a]	22.2 ± 6.5[a]	85.0 ± 4.2[a]
	21	8.4 ± 2.4	19.2 ± 2.6	66.0 ± 2.9[a]
PRHH	1	7.1 ± 3.1[b]	19.1 ± 3.9[b]	89.0 ± 5.5[a,b]
601.1	4	8.3 ± 4.4	19.2 ± 2.8	86.7 ± 5.9[a]
	7	5.7 ± 2.3	28.8 ± 1.9[a]	88.2 ± 4.3[a,b]
	10	7.3 ± 1.3[b]	31.3 ± 4.3[a,b]	88.8 ± 4.4[a]
	14	6.8 ± 0.8[b]	30.6 ± 1.5[a]	81.8 ± 4.8[a]
	21	3.5 ± 1.6[a]	28.6 ± 4.6[a]	64.8 ± 4.8[a,b]

[a] significance of difference $p < 0.05$ from controls.

[b] significance of difference $p < 0.05$ from API #1.

TABLE 3

COMPARISONS OF CELL YIELDS, PERCENTAGES OF CYCLING CELLS AND PERCENTAGE VIABLE CELLS FROM OZONE-EXPOSED RATS

	Elapsed Time (Days)	Total Number of Cells ($\times 10^6$)	Percent Cycling Cells	Percent Viable
trol		1.9 ± 0.7	16.2 ± 4.5	81.3 ± 7.0
ne	2	1.8 ± 0.7	16.0 ± 1.3	68.6 ± 5.7[a]
	3	1.6 ± 0.6	14.9 ± 2.3	78.3 ± 7.0[a]
	4	1.7 ± 0.5	16.6 ± 2.9	74.2 ± 5.2[a]
	7	1.8 ± 0.7	15.1 ± 3.9	68.6 ± 14.2[a]

gnificance of difference $p < 0.05$ from controls.

TABLE 4

PERCENTAGES OF CYCLING CELLS DETERMINED BY FCM ANALYSIS

OF HO STAINED VIABLE TRACHEAL CELLS FOLLOWING OZONE EXPOSURE

Group	Percent Cycling Cells
Control	9.3 ± 0.6[a]
T_1	11.3 ± 0.3
T_2	10.4 ± 1.0
T_3	10.6 ± 0.5
T_6	11.7 ± 0.9
T_8	12.2 ± 0.8
T_{10}	14.2 ± 1.4

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a = Std error of the mean

FIGURE LEGENDS

.gure 1. Light micrographs of skin 14 days post-treatment: A) Cage controls; B) Abraded; C) API #1-treated; D) PHRR-treated. The cage controls and API#1 have a similar normal histological appearance while the abraded skin and the PHRR-treated skins are hyperplastic.

.gure 2. Light micrographs of tracheal epithelial lining: A) Control; B) Two days post-treatment; C) 10 days post-treatment. The 2 day post-treatment animal shows a generalized loss of ciliated cells while there is a hyperplastic/ hypertrophic response in the 10 day post-treatment animal.

.GURE 3. Incidence and latency of skin tumors occurring in animals chronically exposed by epidermal application of the shale oil PHRR and the standard petroleum API #1. It shows the increased tumongenicity of PHRR over API #1.

gure 4. Histograms of DNA content showing the number of events (cells) on the y-axis and fluorescence intensity (DNA) on the x-axis. The first peak is DNA associated with resting cells (G_1); the area between this and the second peak is associated with cycling cells in S-phase; and the second peak are cells in G_2M. These are DNA measurements made on samples 4-days post-exposure: A) Control skin; B) PHARR Shale oil-exposed; C) API#1-exposed; and D) Abraded skin. Note the appearance of excess DNA at the arrow in B.

A

B

C

D

Figure 1

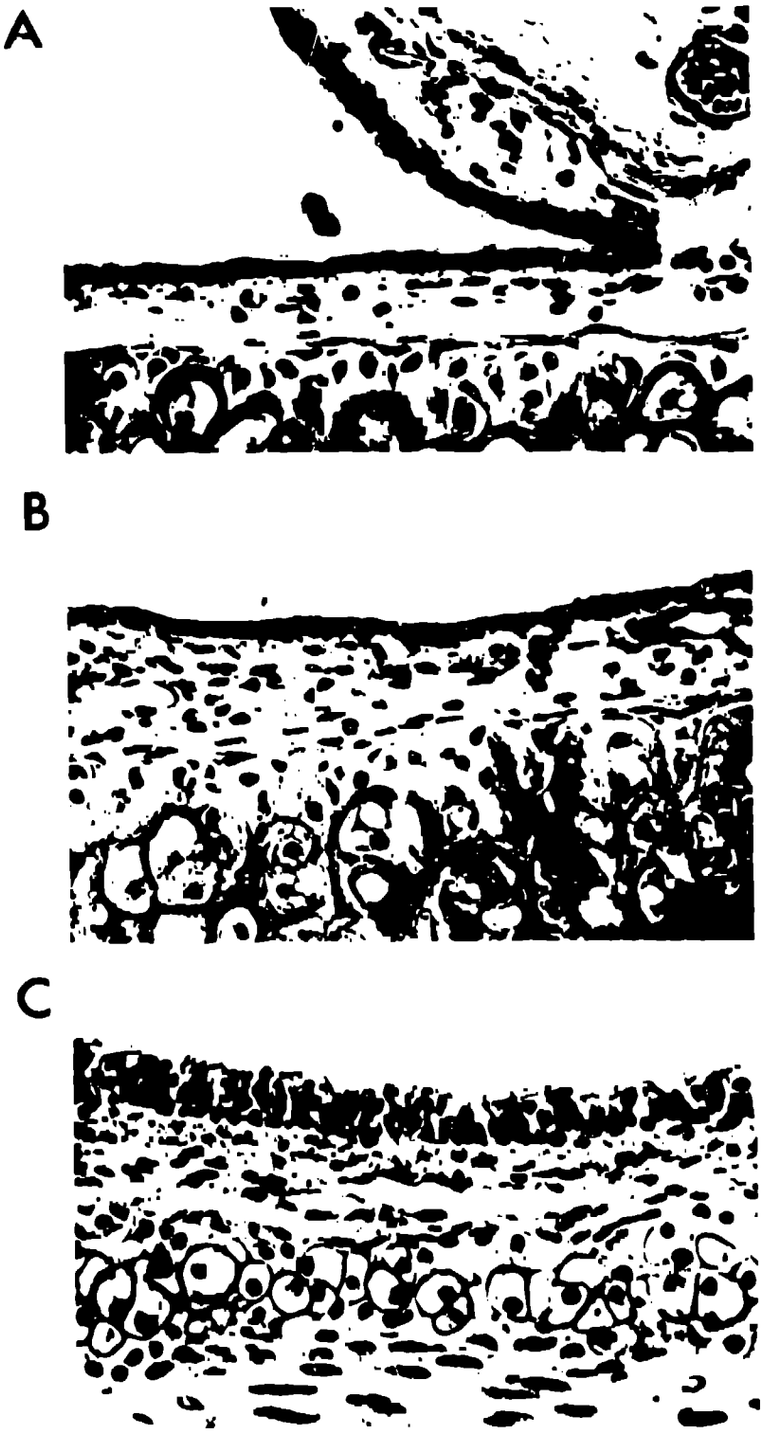

Figure 2

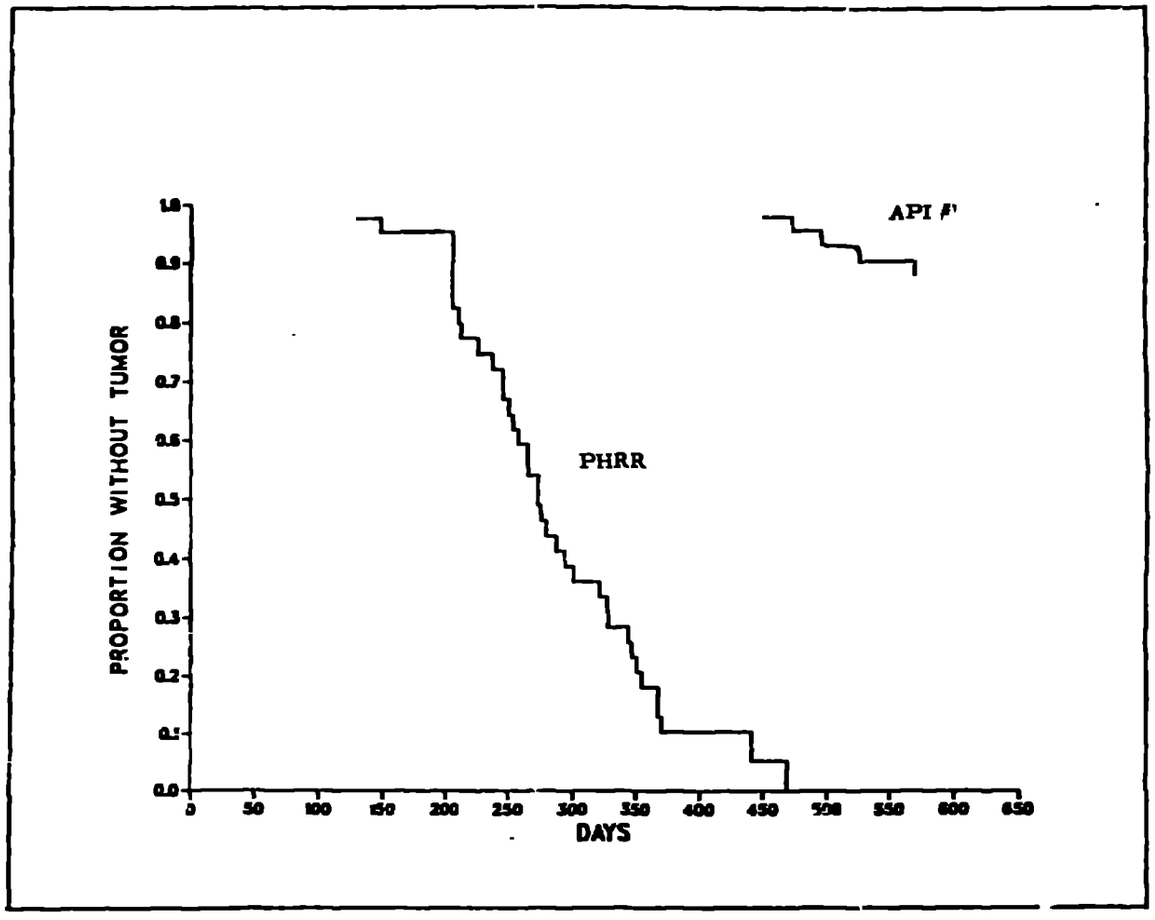

Figure 3

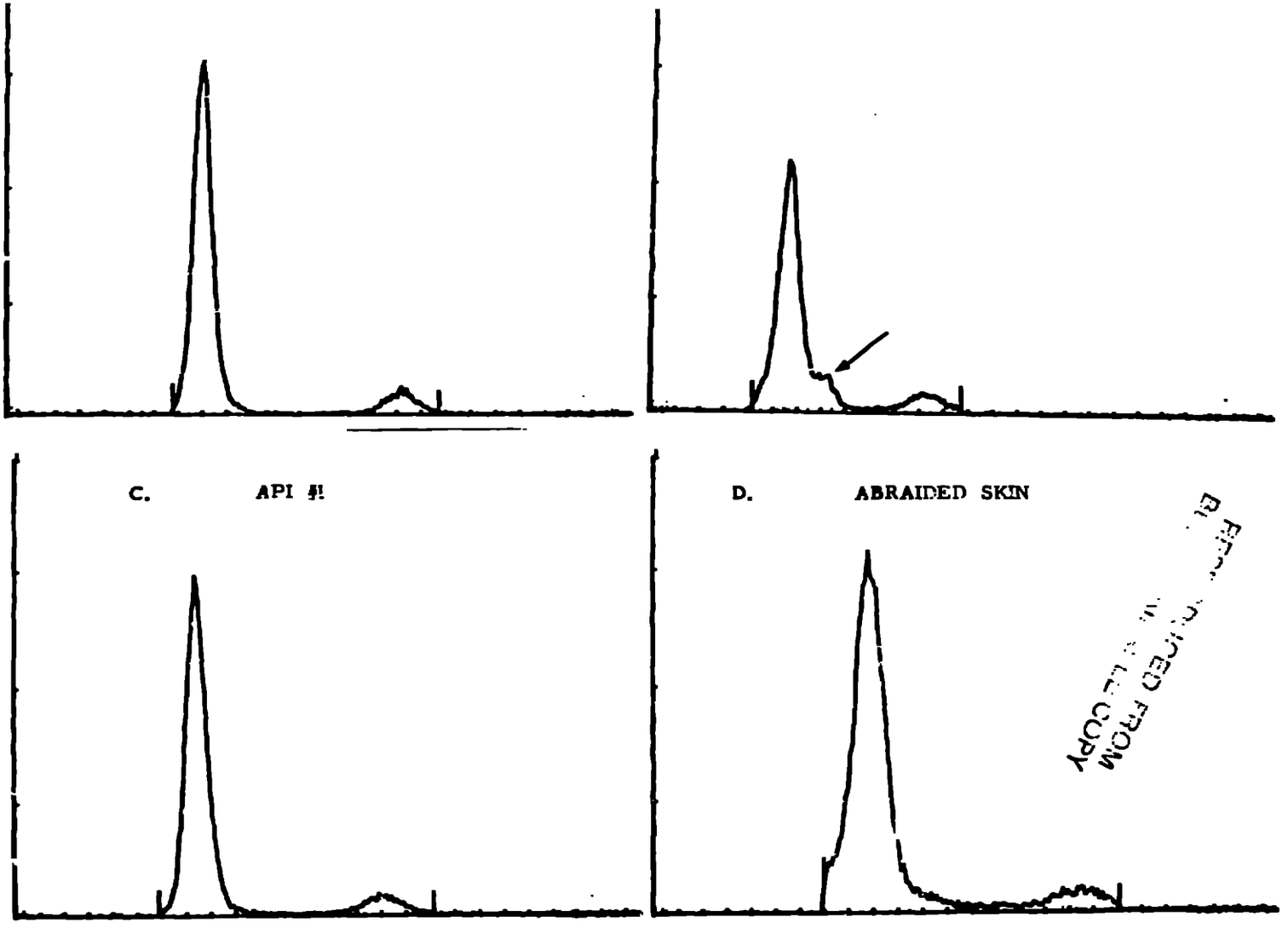

Figure 4